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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29,1998 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Daishiro Miura, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1990 and a Ph.D. degree in 1999 from Hokkaido University;

THAT I am a member of several Japanese Scientific Societies related to research in toxicology, hematology, vitamin D and bone and mineral metabolism.

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1990, in the Safety Research Department, where I have been involved in the toxicity study of pharmaceuticals and biological activities of Vitamin D_3.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the final Office Action of June 12, 2001 issued in reference to the application. In response to the final Office Action, I submit herewith this Declaration comparing the potency of 20(S)-forms versus 20(R)-forms of Vitamin D derivatives and as evidence that the 20(S)-forms are significantly more potent than the 20(R)-forms in their ability to induce cellular differentiation.

Comparison of Activity for 20(S)- versus 20(R)-forms of Vitamin D derivatives for induction of HL-60 cell differentiation.

Materials and methods.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passed by culturing. Cells which had been treated by successive culturing for one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum (FBS). Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well.

An ethanol solution containing the 20(S)-form compounds, (68), (71), (72) or (74), at concentrations ranging from 1 x 10^{-9} M to 1 x 10^{-6} M (for Table 1) or 1 x 10^{-7} M to 1 x 10^{-4} M (for Table 2) was added to each well at 1 µl/well. Further, regarding 1α,25-$(OH)_2D_3$, an ethanol solution containing 1 x 10^{-7} M to 1 x 10^{-4} M of the compound was added at 1 µl/well, and for the control, ethanol alone was added at 1 µl/well.

An ethanol solution containing the 20(R)-form compounds, (3), (4), (6) or (65), at concentrations ranging from 1x10^{-7} M to 1x10^{-4} M was added to each well at 1 µl/well. For the control, ethanol alone was added at 1 µl/well.

After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reducing activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min. Then, cells were collected by centrifugation , resuspended in FBS and cytospin smears were prepared. After air-drying, smears were stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reducing) was determined under an optical microscope.

Results.

The results are shown in the following Table1 (Comparison of activity for 20(S)-form compounds at concentrations ranging from 1 x 10^{-12} M to 1 x 10^{-9} M and 20(R)-form compounds at concentrations ranging from 1 x 10^{-10} M to 1 x 10^{-7} M) and Table 2 (Activity for 20(S)-form compounds at concentrations ranging from 1 x 10^{-10} M to 1 x 10^{-7} M). These results shown in Table 1 and Table 2 were obtained from independent two experiments.

Table 1.

Sample	Concentration (M)	% Cells showing NBT activity	Sample	Concentration (M)	% Cells showing NBT activity
Control		1.5	Control		5.8
$1\alpha,25\text{-}(OH)_2D_3$	10^{-10} 10^{-9} 10^{-8} 10^{-7}	4.3 ± 1.2 36.8 ± 2.0 86.1 ± 2.6 96.5 ± 1.0			
20(S)-form			**20(R)-form**		
Compound (68) [1]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	1.7 ± 0.3 2.8 ± 0.7 57.7 ± 5.0 95.7 ± 1.0 – –	Compound (65) [2]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 1.8 ± 0.7 1.4 ± 0.8 32.0 ± 3.2 95.1 ± 2.8
Compound (71) [3]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	1.5 ± 0.8 1.8 ± 0.8 2.0 ± 1.0 40.5 ± 1.8 – –	Compound (3) [4]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 1.6 ± 1.1 1.3 ± 0.5 1.8 ± 0.7 1.4 ± 0.2
Compound (74) [5]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	6.4 ± 1.1 17.0 ± 2.3 16.7 ± 1.1 96.4 ± 1.4 – –	Compound (6) [6]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 1.9 ± 0.6 1.8 ± 0.2 12.9 ± 2.2 92.7 ± 2.9
Compound (72) [7]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	3.7 ± 0.8 94.4 ± 1.8 95.7 ±2.3 96.2 ±2.0 – –	Compound (4) [8]	10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}	– – 2.3 ± 0.9 69.4 ± 3.9 93.9 ± 4.4 95.9 ± 1.1

[1] Example 2, page 33 of the specification.
[2] Page 40, line 1 of the specification.
[3] Example 5, page 36 of the specification.
[4] Page 39, line 30 of the specification.
[5] Example 7, page 37 of the specification.
[6] Page 39, line 33 of the specification.
[7] Example 1, page 32 of the specification.
[8] Page 39, line 31 of the specification.

Table 2.

Sample	Concentration (M)	% Cells showing NBT activity
Compound (68)	10^{-10}	90.8
	10^{-9}	98.3
	10^{-8}	98.0
	10^{-7}	99.5
Compound (71)	10^{-10}	22.0
	10^{-9}	47.1
	10^{-8}	97.5
	10^{-7}	97.7
Compound (74)	10^{-10}	52.1
	10^{-9}	95.4
	10^{-8}	97.7
	10^{-7}	98.7
Compound (72)	10^{-10}	93.7
	10^{-9}	97.9
	10^{-8}	98.6
	10^{-7}	98.6

Conclusions.

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20(S)-forms show excellent efficacy compared to the 20(R)-forms. Comparison of compound (68) with compound (65); compound (71) with compound (3); compound (74) with compound (6) and compound (72) with compound (4) reveals that the 20(S)-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: November 7, 2001

Daishiro Miura

Daishiro Miura